

Reversal of Neuropathology and Motor Dysfunction in a Conditional Model of Huntington's Disease

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Summary

Neurodegenerative disorders like Huntington's disease (HD) are characterized by progressive and putative irreversible clinical and neuropathological symptoms, including neuronal protein aggregates. Conditional transgenic models of neurodegenerative diseases therefore could be a powerful means to explore the relationship between mutant protein expression and progression of the disease. We have created a conditional model of HD by using the tet-regulatable system. Mice expressing a mutated huntingtin fragment demonstrate neuronal inclusions, characteristic neuropathology, and progressive motor dysfunction. Blockade of expression in symptomatic mice leads to a disappearance of inclusions and an amelioration of the behavioral phenotype. We thus demonstrate that a continuous influx of the mutant protein is required to maintain inclusions and symptoms, raising the possibility that HD may be reversible.

Introduction

Huntington's disease (HD) is an autosomal dominant inherited disorder that is progressive (Wexler et al., 1987). Typically, HD manifests in midlife, and death follows 10 to 20 years after onset. The disorder is characterized by motor disturbances such as chorea and dystonia, personality changes, and cognitive decline (Ambrose et al., 1994). The pathology is restricted to the brain, with atrophy occurring foremost in the striatum and to a lesser extent in the cortex. Neither a specific treatment nor a cure is yet available.

HD is caused by an expansion of CAG repeats (Rubinsztein et al., 1994; Gusella and MacDonald, 1996) near the 5' end of the IT15 gene (HDCRG, 1993). IT15 encodes a ubiquitously expressed 350 kDa protein of unknown function called huntingtin (htt) (Ambrose et al., 1994). The CAG repeats are translated into a polyglutamine (polyQ) sequence in the N-terminal portion of htt. While normal individuals possess a polyQ length of 6 to 34 repeats, individuals with greater than 40 repeats develop HD with virtually 100% penetrance. An inverse correlation exists between repeat length with the severity of symptoms and age of onset (Persichetti et al., 1995).

The pathogenesis of the polyQ expansion on htt function is poorly understood. A loss of function is unlikely, as heterozygous and homozygous patients with the mutation have similar clinical features (Wexler et al., 1987). Further, MacDonald and colleagues have identified an individual that showed no abnormal phenotype despite a 50% reduction in expression of the normal gene product due to a deletion in IT15 (Ambrose et al., 1994). Similarly, mice heterozygous for the htt null mutation do not show features of HD, and homozygotes die early in embryogenesis (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). A deleterious gain-of-function mechanism is therefore more probable. This is supported by the observation that polyQ stretches exceeding similar thresholds are responsible for several neurological disorders including spinobulbar muscular atrophy (SBMA), dentatorubral-pallidoluysian atrophy (DRPLA), and the spinocerebellar ataxias (SCA) 1, 2, 3, and 7 (Ross, 1997). These disorders, known as CAG-repeat disorders, share many common traits such as a progressive phenotype (Gusella and MacDonald, 1996), subcortical and cortical atrophy (Koshy and Zoghbi, 1997), as well as neuronal nuclear aggregates (Davies et al., 1997; DiFiglia et al., 1997; Becher et al., 1998).

Transgenic mice have been generated to understand the pathogenesis of HD. The models vary in the promoter used, the length of the htt protein, and in the length of the polyQ (Mangiarini et al., 1996; White et al., 1997; Reddy et al., 1998; Hodgson et al., 1999; Schilling et al., 1999; Usdin et al., 1999). Nevertheless, all transgenic mice that express an abnormal polyQ length demonstrate a progressive HD-like phenotype while transgenic mice with a normal polyQ length do not. The most extensively characterized of these models, the Bates R6 lines (Mangiarini et al., 1996), reveal that expression of only exon 1 containing a polyQ expansion is sufficient to produce mice with essential neuropathological features of HD. Included among these features are a decrease in striatal and total brain size (Hansson et al., 1999), changes in neurotransmitter receptor levels (Cha et al., 1998, 1999), and nuclear and cytoplasmic aggregates (Davies et al., 1997; Li et al., 1999). These transgenic mice also develop a progressive motor decline including dystonic and choreic-like movements and late-stage hypoactivity (Carter et al., 1999).

Despite the insights gained from the HD mouse models, many critical questions remain. Is the cascade of pathologic events initiated by the mutant protein dependent upon its continuous expression? Can the disease be treated after onset of neuropathology or motor symptoms, or is the damage irreversible? Finding the answer to these questions can have profound implications for the development of a therapy for HD. We therefore have created a conditional mouse model of HD using the tetracycline-responsive gene system. This system allows expression of a transgene to be turned off with oral administration of tetracycline analogs (Furth et al., 1994; Kistner et al., 1996). Our results confirm that expression of a truncated N-terminal htt with an abnormal polyQ stretch (polyQ-htt) can generate core neuropathological

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and behavioral deficits of HD. More importantly, we demonstrate that the progression of the HD-like pathology is dependent upon continuous expression of the polyQ-htt. Furthermore, we find that abolishing expression of the mutant protein not only halts progression of the disease, but can reverse aggregate formation and progressive motor decline.

Results

Mouse Design

The tet-regulated system has been used for conditional expression in eukaryotic cell systems and mice (Furth et al., 1994; Kistner et al., 1996; Mayford et al., 1996; Freundlieb et al., 1997). Regulation of the system is achieved through the tetracycline-regulated transactivator (tTA), a fusion protein between the tet-repressor binding domain and a VP16 activation domain (Gossen and Bujard, 1992). This protein binds specifically to the tetO operator and induces transcription from an adjacent CMV minimal promoter. The combination of both tTA and the tetO elements allows for continual expression of a given transgene. Tetracycline and its analogs can bind to tTA. When this happens, tTA is prevented from binding to tetO and transcription is inhibited.

We generated transgenic mice carrying the bidirectional tetO responsive promoter (Baron et al., 1995) followed by both a chimeric mouse/human exon 1 with a polyQ expansion of 94 repeats in one direction and β -galactosidase (lacZ) reporter sequences in the other (Figure 1A). These mice are designated BiTetO. The tTA transgene used is under the control of the calcium/calmodulin kinase II α promoter (CamKII α -tTA line E and B) (Mayford et al., 1996). These tTA lines were chosen to allow for restricted, conditional expression in the CNS, with particularly high expression in the forebrain (Mayford et al., 1996; Ghavami et al., 1999). When the BiTetO mice are crossed with CamKII α -tTA mice, the resulting double transgenic progeny (designated HD94) constitutively express both transgenes (Figure 1B). This expression, however, can be abolished in the presence of doxycycline (dox).

Heterozygote crosses between BiTetO and CamKII α -tTA mice did not yield the expected frequency of 25% for each genotype (wt, BiTetO, tTA, and HD94). The HD94 mice were underrepresented (6%), because of a perinatal lethality probably due to expression of the polyQ-htt. In order to ensure that expression of the polyQ-htt was postnatal, pregnant mice were given dox (2 mg/ml) in their drinking water ad libitum beginning at E15. After birth, the dox solution was removed. The prenatal dox administration allowed for a Mendelian distribution of the four genotypes. All mice in this study thus received dox from E15 to P0. Crosses with both tTA-expressing lines, CamKII α -tTA line E and B, produced similar results.

Expression of polyQ-htt

LacZ was used to assess the expression pattern and effectiveness of this conditional system (Figures 2 and 5). In the adult brain of double transgenic HD94 mice four weeks of age and older, a high level of lacZ staining

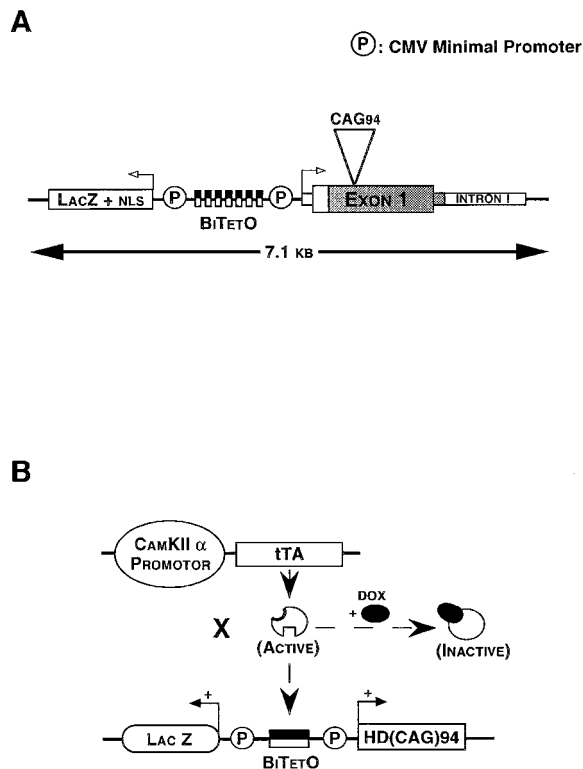


Figure 1. Mouse Model Design

(A) Inducible polyQ-htt transgene: The mutant huntingtin peptide consists of a chimeric mouse/human exon 1 with a polyQ expansion of 94 repeats (HD[CAG]94, see Experimental Procedures). BiTetO will allow for simultaneous regulation of lacZ with a nuclear localization sequence (NLS).

(B) The tTA molecule is driven by the CamKII α promoter. In its active form, tTA binds to the BiTetO sequence to drive expression of both transgenes. Doxycycline can inhibit expression of the transgenes by binding to tTA, rendering it inactive.

was seen in the striatum, septum, cortex, and hippocampus (Figures 2B and 2C and not shown). Expression also was found to a lesser extent in amygdala and hypothalamus, while no expression was detected in the cerebellum (data not shown). The lacZ expression patterns appeared similar to those obtained from crosses between the CamKII α -tTA lines and other tetO reporter lines (Ghavami et al., 1999). Single transgenic BiTetO mice showed no staining in any region (Figure 2A), thus demonstrating that the CMV minimal promoters remained silent in the absence of the tTA protein.

Analysis of the expression of polyQ-htt was performed with an antibody raised against an N-terminal sequence of htt, which recognizes the native protein as well as the aggregates characteristic of the mutant protein (Davies et al., 1997; Scherzinger et al., 1997). Eight-week-old wt ($n = 4$), single transgenics BiTetO ($n = 3$) and CamKII α -tTA ($n = 3$), and double transgenic HD94 ($n = 4$) mice were examined. Increased immunoreactivity was found in HD94 mice in the striatum, cortex, hippocampus (Figures 2E and 2F), and all other lacZ-positive regions (data not shown). Similarly, regions that were lacZ negative exhibited no increased staining for the polyQ-htt, as was the case for the wt and single transgenic controls (Figure 2D).

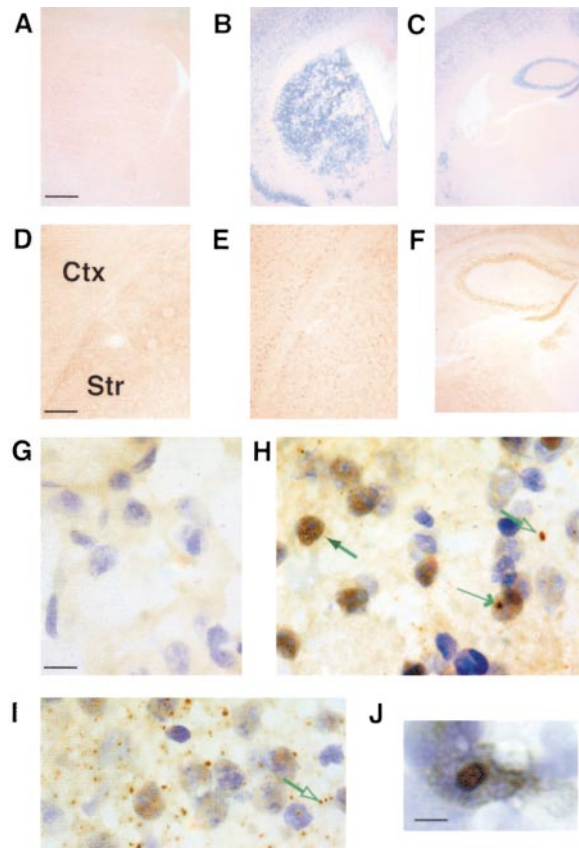


Figure 2. LacZ and polyQ-htt Expression

(A–C) LacZ expression was determined in coronal brain sections. Tissue was counterstained with fast red. (A) BiTetO single transgenic controls were negative for lacZ. (B and C) HD94 mice demonstrated lacZ positive staining in striatum and cortex (B) and the hippocampus (C). (D–F) Sections serial to those stained for lacZ were examined for polyQ-htt expression using an antibody raised against an N-terminal htt (Davies et al., 1997). (D) BiTetO sections were not immunopositive for polyQ-htt (E and F) HD94 brains showed polyQ-htt immunoreactivity in all regions positive for lacZ expression, such as the cell layer of the hippocampus (C and F), but not in those regions that were negative. (G–J) Nuclear and extranuclear staining for polyQ-htt was determined using an N-terminal htt antibody. Sections were counterstained with cresyl violet to visualize nuclei. (G) No specific staining was detected in striatum from BiTetO single transgenic control sections. (H and J) Specific staining in the striatum of double transgenic HD94 mice was mostly diffuse and nuclear (sharp arrowhead), with distinct nuclear aggregates of various sizes (short arrowhead and J), and few extranuclear aggregates (open arrowhead). (I) Staining in the cortex of HD94 mice demonstrated a higher degree of extranuclear, neuritic-like staining (open arrowhead). Scale bars: (A–C) 1 mm; (D–F) 500 μ m; (G–I) 100 μ m; (J) 50 μ m.

Higher magnification revealed that the majority of striatal cells displayed immunoreactivity (Figures 2G, 2H, and 2J). The most common immunoreactive pattern was a diffuse nuclear staining (Figure 2H, sharp arrow). Nuclear aggregates of different sizes (Figures 2H, blunt arrow; and 2J) and, to a lesser extent, extranuclear aggregates (Figure 2H, open arrow) were also found. The immunoreactivity seen in the cortex was similar to the striatum. However, the occurrence of extranuclear aggregates was much more frequent (Figure 2I). These

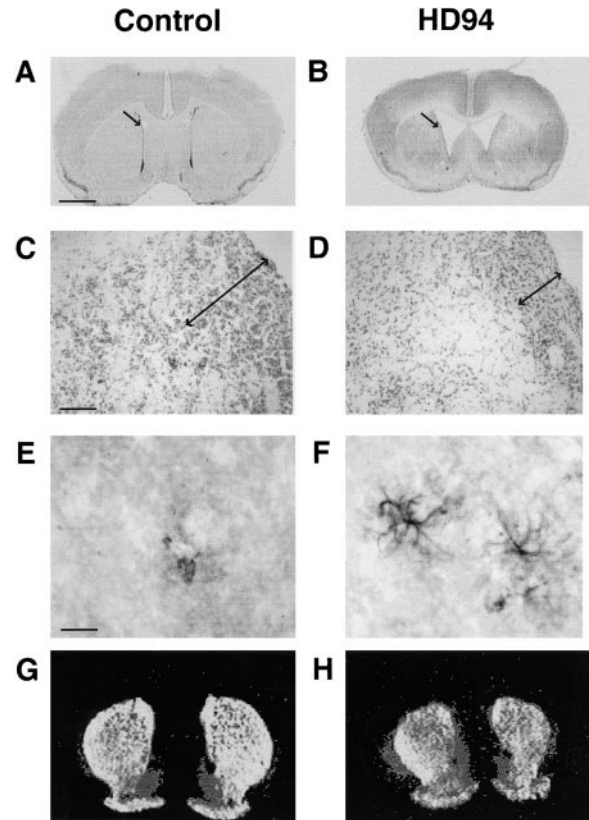


Figure 3. Neuropathology of 18-Week-Old HD94 Mice

Neuropathological changes were evident in brains of double transgenic HD 94 mice (B, D, F, and H) versus single transgenic BiTetO control mice (A, C, E, and G). For quantification, see Figure 6. (A and B) Nissl staining revealed a decrease in total brain and striatal size, and an increase in ventricular size for HD94 mice. (C and D) Enlargement of the area indicated by the arrows in (A) and (B). The ventricular zone is narrower in HD94 mice (double-headed arrow). (E and F) Reactive astrocytosis in the striatum of HD94 mice as indicated by GFAP staining. (F) Reactive astrocytes with the characteristic hypertrophied star-like shape. (E) GFAP-positive cells in BiTetO control mice were less abundant and were not reactive. (G and H) D1 receptor levels were detected using radioligand binding with [3 H]-SCH23380. Images are presented in inverted gray-scale. (H) HD94 mice had significantly lower receptor levels. The difference was greater in the medial striatum than the lateral striatum. Scale bar: (A and B) 1 mm; (C and D) 500 μ m; (E and F) 500 μ m.

aggregates resembled neuritic staining and appeared singly or in multiples much like a string of beads (Figure 2I, open arrow). Staining with an antibody raised against ubiquitin revealed a similar pattern, although the diffuse intranuclear staining was less prevalent (data not shown).

Neuropathology in HD94 Mice

We analyzed neuropathological changes apart from aggregates (Figure 3, for quantitative results see Figure 6). Upon examination of coronal sections of the brains in 18-week-old HD94 mice, two differences were immediately apparent. First, the HD94 brains were smaller than their age-matched controls (Figures 3A and 3B). Second, a decrease in striatal size accompanied by a gross enlargement of the ventricles was evident. Nissl staining

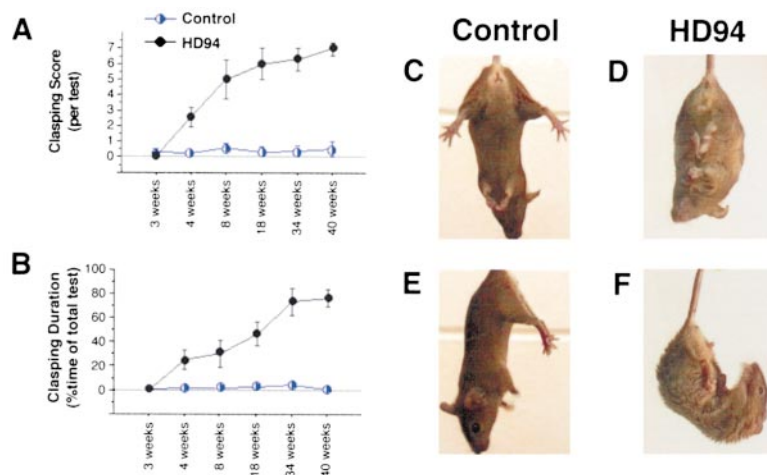


Figure 4. Progressive Clasping Phenotype of HD94 Mice

Mice were observed during a 15 s tail suspension test to monitor the progression of the clasping phenotype between 3 and 40 weeks of age. Mice were analyzed for (A) clasping score and (B) clasping duration (represented in percent control). Control mice represent a pool of BiTetO, tTA and wt genotypes. No difference was detected between these three genotypes. (C and E) 40-week-old BiTetO mouse during the tail suspension test, and (D and F) an age-matched HD94 mouse displaying a characteristic full body clasp.

revealed that the ventricular zone of the striatum was noticeably narrower (Figures 3C and 3D).

Reactive astrogliosis in the striatum was also evident in the HD94 mice. Staining with antibodies raised against glial fibrillary acid protein (GFAP) revealed a higher incidence of staining in the striatum than in control mice. These astrocytes were clearly reactive (Figure 3F) and were hypertrophied with the characteristic star-like shape. The number of reactive astrocytes increased over time in HD94 mice, and the gliosis spread throughout the lateral and medial striatum. In contrast, only very small GFAP-positive astrocytes were seen in control brains (Figure 3E). These positive cells were nonreactive astrocytes, because the GFAP staining was not as robust as the reactive hypertrophied astrocytes observed in HD94 brains (Ridet et al., 1997).

A decrease in D1 receptors has been described in HD patients (Turjanski et al., 1995). We therefore assessed the levels of these dopamine receptors using an autoradiographic binding assay with [3 H]-SCH 23969. D1 receptor levels were significantly decreased in the striatum of HD94 mice (Figure 3H). This decrease was not uniform, manifesting more severely from medial striatum to lateral.

Behavioral Phenotype

HD is characterized by a progressive motor dysfunction with choreic and dystonic movements, and parkinsonism. The most common dysfunction demonstrated in the available genetic models is a progressive clasping of the limbs, which is triggered by a tail suspension test (Mangiarini et al., 1996; Reddy et al., 1998; Carter et al., 1999; Schilling et al., 1999). Mice were suspended by the tail for 15 s every 2 weeks between the ages of 3 and 40 weeks. An abnormal movement was scored when the mouse moved its hindlimbs, forelimbs, or trunk in a dystonic fashion. Both the score and duration of the abnormal movement were recorded (Figures 4A and 4B). Until 4 weeks of age, double transgenic HD94 mice were indistinguishable from control littermates in this test. At 4 weeks, some HD94 mice began to clasp, and by 8 weeks all were clasping. At onset, the phenotype involved only the hindlimbs, which were curled into the body in a dystonic fashion and held and released in

short bursts. Both score and duration of the clasping increased over time. As early as 28 weeks, mice began to show a full body clasp. By 40 weeks, all mice immediately assumed a full body clasp and maintained this for the entire duration of the test (Figures 4D and 4F). In many cases, the posture was not immediately released when the mice were returned to their cage.

In addition to clasping, at 20 weeks HD94 mice began to show a mild tremor that developed into a jerking motion in some, but not all, mice. Onset of tremor was accompanied by decreased grooming, and the fur of older HD94 mice appeared unkempt (Figure 4F). BiTetO single transgenic mice were indistinguishable from wt and did not exhibit the clasping or tremor phenotypes.

In other assessments, HD94 animals did not greatly differ from their control littermates. Testing in the open-field paradigm at 8 weeks demonstrated no difference in total locomotion (data not shown). Nonetheless, by 36 weeks, HD94 mice were clearly hypoactive, and remained so until death. Adult HD94 mice were fertile, but as the clasping and tremor worsened, the frequency of mating by males decreased. No atrophy or deformation was evident in the testes of 32-week-old mice. Finally, although the average life span of double transgenics was similar to that of wt (2 years), a small number of HD94 mice died earlier (8 to 10 months). These mice appeared to have died by sustaining severe injuries from fighting with littermates.

Evolution of Neuropathology and Behavior after Turning off polyQ-htt Expression

To analyze the evolution of the phenotype in the absence of polyQ-htt, transgene expression was turned off with doxycycline (dox). Dox treatment began at 18 weeks, an age at which the neuropathology and motor behavior disruption were evident. Mice were split into two groups. The "gene-on" group was allowed to develop without intervention, and the "gene-off" group was treated with 2 mg/ml of dox in their drinking water (Figure 5G). Treatment was maintained for 16 weeks.

LacZ staining revealed that the dox regimen completely abolished expression of the transgene (Figures 5A and 5B). Tissue sections were next examined for htt and the percentage of neurons positive for nuclear htt

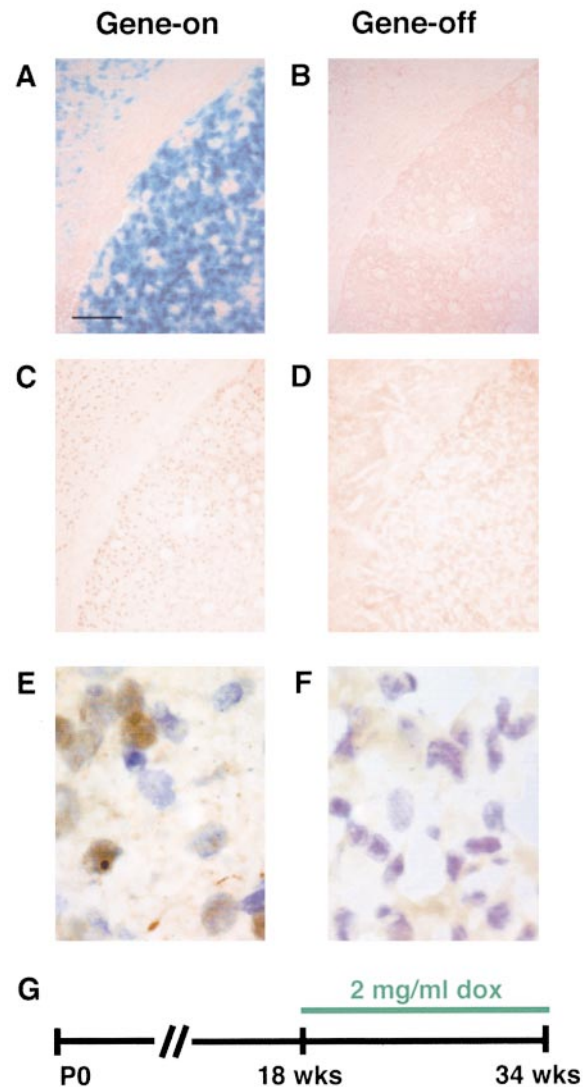


Figure 5. LacZ and htt Aggregate Staining after 16 Week "Gene-Off" Period

18-week-old HD94 mice were given either 2 mg/ml dox ("gene-off") or vehicle ("gene-on") for 16 weeks (see G for schematic). (A and B) LacZ expression was abolished in gene-off HD94 mice and maintained in age-matched HD94 gene-on mice.

(C and D) Images show cortex and striatum as seen in Figure 2. Immunostaining for htt also was drastically reduced in gene-off mice. (C) Immunopositive staining is present in the cortex and striatum of gene-on mice. (D) In age-matched gene-off mice, the staining in the striatum was undetectable, while staining in the cortex was confined to scattered cells.

(E and F) Higher magnification of the striatum counterstained with cresyl violet revealed that the lack of immunoreactivity in gene-off mice corresponds to a disappearance of both intra- and extranuclear aggregates.

Scale bars: (A–D) 500 μ m; (E and F) 100 μ m.

was assessed. At 18 weeks, 39.9% of the striatal neurons were positive for nuclear htt while at 34 weeks, the value increased to 60.0%. In sharp contrast to the gene-on HD94 mice, less than 1% of the striatal neurons from the gene-off HD94 mice demonstrated nuclear htt (Figures 5C and 5D). A similarly drastic reduction was

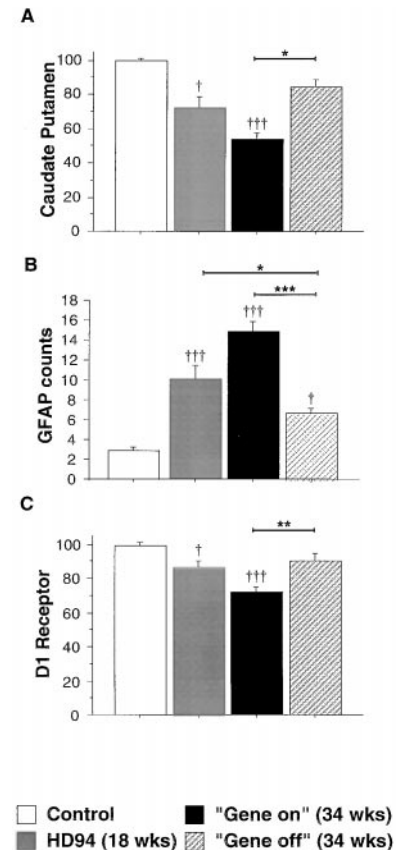


Figure 6. The Effect of a 16 Week "Gene-Off" Period on the Neuropathological Phenotype of HD94 Mice

Brains of 18-week-old HD94 mice were compared with 34-week-old gene-on and 34-week-old gene-off HD94 mice. Repeated-measures ANOVA was conducted across matched sections from brains from each group. Single transgenic and wt mice at age 18 weeks and 34 weeks did not significantly differ in any of the measurements, and have thus been pooled as control.

(A) Area of caudate putamen; main effect of group $F_{(3,8)} = 9.666$; $P < 0.005$.

(B) Counts for GFAP-positive reactive astrocytes; main effect of group $F_{(3,8)} = 14.330$; $P < 0.005$.

(C) D1 receptor binding levels are expressed in percent control mean density; main effect of group $F_{(3,8)} = 56.515$; $P < 0.005$.

Significant differences (Fisher post-hoc) between one HD94 group and control are marked by "†": †, $P < 0.05$; ††, $P < 0.005$; and from "gene-off" (34 weeks) are marked by *: *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.005$.

seen in the cortex, though to a lesser extent. Further analysis of the striatal tissue at higher magnification revealed that not only diffuse nuclear staining was reversed, but both intra- and extranuclear aggregates disappeared. The remaining cortical staining corresponded to fewer diffuse nuclear stainings and small extranuclear aggregates (data not shown).

A quantitative neuropathological determination revealed a clear arrest of neuropathology in the gene-off mice. Compared to controls, striatal area was significantly smaller in 18-week-old HD94 mice and showed a further significant decrease at 34 weeks (Figure 6A). On the other hand, gene-off mice did not show further reduction in striatal size while treated with dox. At 34

weeks, the striatal size of these gene-off mice was significantly larger than the striatal size of 34 week gene-on mice, and not significantly different from 18-week-old mice. GFAP-positive astrocytes were also counted (Figure 6B). While the number of reactive astrocytes increased in the gene-on HD94 mice, the number significantly decreased in the gene-off mice. As a result, the number of astrocytes in the gene-off mice was intermediate between 18-week-old HD94 mice and control.

A change in the D1 receptor level was also seen. By 18 weeks, D1 receptor levels were significantly lower in HD94 than in control animals, and the levels continued to decrease over time (Figure 6C). Shutting off expression of the polyQ-htt halted this progressive loss. The receptor levels in the gene-off HD94 mice were higher than the age-matched gene-on HD94 mice, but did not significantly differ from the levels obtained in 18-week-old HD94 brains.

Reversal of the Motor Dysfunction

Shutting off expression of the mutant transgene produced significant changes in the neuropathological phenotype of the HD94 mice. We therefore examined the progressive claspings phenotype in mice after the gene-off period. Naive mice were assessed for claspings at 18 weeks. They were then split into a gene-on group and a gene-off group (receiving water and dox treatment, respectively). At 34 weeks, these mice were reassessed for claspings (Figure 7).

In the gene-on group, there was an increase in the score for claspings and a significant increase in the duration of claspings when expression of the polyQ-htt was maintained between 18 and 34 weeks. In contrast, the gene-off group showed a significant decrease in both the score and duration of claspings movements. The score for claspings reversed to a level comparable to double transgenics at 8 weeks of age, while the duration of claspings reversed to levels similar to that of control mice. The dox treatment did not affect the single transgenic control mice (data not shown).

Discussion

Using the tetracycline-responsive gene system, we have created a conditional mouse model of a neurodegenerative disorder. We found that suppression of polyQ-htt between the ages of 18 and 34 weeks either halted or reversed the different aspects of the HD-like phenotype in the HD94 mice. Specifically, the nuclear and cytoplasmic aggregates disappeared, the number of reactive astrocytes decreased, and the progressive striatal atrophy and decrease in D1 receptor levels halted. Furthermore, stopping expression of the polyQ-htt not only prevented the worsening of the claspings phenotype, but also ameliorated it to levels approaching the control.

Expression of polyQ-htt under the control of a forebrain-specific promoter was sufficient to create a mouse model of Huntington's disease (HD94). The phenotype of these mice is consistent with HD patients: an abnormal motor phenotype (Martin and Gusella, 1986), a decrease in striatal size (Aylward et al., 1994; Backman et al., 1997; Halliday et al., 1998), intra- and extranuclear aggregates

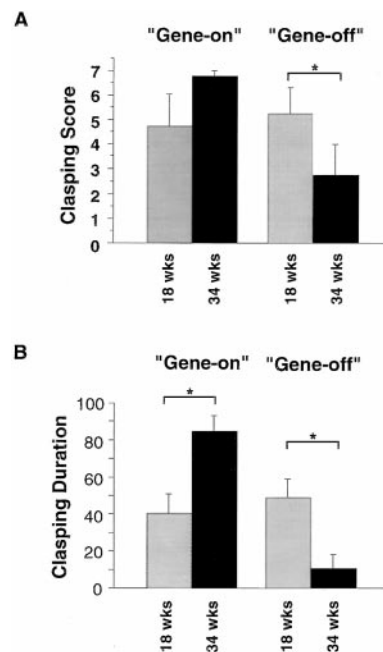


Figure 7. Inhibiting Expression of polyQ-htt Allows for Amelioration of the Claspings Phenotype

Naive 18-week-old HD94 mice were assessed in the tail suspension test and then retested after a treatment of either 2 mg/ml dox ("gene-off") or vehicle ("gene-on") for 16 weeks.

(A) Average score per 15 s test. Repeated-measures ANOVA confirmed an interaction between claspings score and age $P < 0.05$, $F_{(1,6)} = 11.571$. Post-hoc Fisher analysis revealed that in the gene-off group, the HD94 34-week-old mice scored significantly lower than the HD94 18-week-old mice (*, $P < 0.05$).

(B) Percent of total test time spent in clasp. Repeated-measures ANOVA confirmed an interaction between claspings duration and age, $P < 0.005$, $F_{(1,6)} = 25.165$. Post-hoc Fisher analysis revealed that in the gene-on group, HD94 34-week-old mice scored significantly higher than HD94 18-week-old, while in the gene-off group, HD94 34-week-old scored significantly lower than HD94 18-week-old (*, $P < 0.05$).

(Alves-Rodrigues et al., 1998), selective reactive astrocytosis in the striatum (Hedreen and Folstein, 1995), and a decrease in D1 receptor levels (Turjanski et al., 1995; Augood et al., 1997). Also, as in HD patients, most of the pathological characteristics increased in severity over time. The phenotype of these mice confirm previous findings that full-length htt is not necessary to recapitulate key characteristics of HD (Mangiarini et al., 1996; Vonsattel and DiFiglia, 1998; Carter et al., 1999; Schilling et al., 1999).

The progressive striatal atrophy in HD patients has been mainly attributed to neuronal loss and has been associated with the occurrence of reactive astrocytosis (Vonsattel et al., 1985; Myers et al., 1991). In the striatum of our HD94 mice, there is a clear indication of selective reactive astrocytosis. However, preliminary cell density measurements have revealed that despite a 25% decrease of the striatal area, no significant difference in neuron number between control striata ($n = 2$ brains, 7253.75 ± 303.35 neurons/ μm^2) and 18 week HD94 striata ($n = 2$ brains, 7347.00 ± 690.90 neurons/ μm^2) can be demonstrated. Well-correlated with this finding is the

very limited number of TUNEL-positive stainings seen in the HD94 mice. An explanation for the reduction in striatal area may be found in the R6 transgenic lines. In both the R6/1 and R6/2 mice, a decrease in striatal volume without a change in cell number has been reported (Mangiarini et al., 1996; Hansson et al., 1999). To account for the volumetric decrease, Hansson et al. hypothesized that the change in volume can be attributed to a reduction in either cell size or in extracellular matrix. In the HD94 mice, it is possible that similar mechanisms are applicable.

Our main intent was to determine if constant expression of the polyQ-htt is necessary for the progression of the various phenotypes. We therefore shut down expression of the transgene at an age when HD94 mice clearly displayed the pathologic phenotype. One of the most dramatic changes seen in the gene-off mice was the disappearance of the aggregates. Biochemical analysis had indicated that the aggregates were extremely stable structures (Davies et al., 1999; Kazantsev et al., 1999). Our results show that despite the difficulty in biochemically dissolving or denaturing them, the neurons are capable of dispelling the nuclear and extranuclear accumulation of htt. Our results suggest that the ubiquitinated aggregates are neither inaccessible to the proteasome, nor an indication that the proteolytic pathways are fully or irreversibly compromised (Lansbury, 1997; Alves-Rodrigues et al., 1998). Our findings also argue against, but do not preclude, models in which polyQ-htt is required only to initiate the process of aggregation of other cellular proteins including wild-type htt. (Lansbury, 1997).

The second significant finding is the reversion of the clasping phenotype. Among the histological measurements we performed on these animals, only two demonstrated reversal when the gene was turned off: aggregate formation and reactive astrogliosis. This correlation between the disappearance of aggregates and the amelioration of the clasping phenotype may suggest that the aggregates are responsible for the pathology. However, it is equally plausible that the aggregates are not toxic themselves, but a consequence of the toxicity of the soluble polyQ-htt. They might therefore act as an indicator of cellular dysfunction or a means by which the neurons attempt to protect themselves (Klement et al., 1998; Saudou et al., 1998). Our experiments do not distinguish between these scenarios, because by abolishing expression of the soluble polyQ-htt, the aggregates also disappeared. However, our results further confirm that neither soluble nor aggregated polyQ-htt causes neurons to die. At the onset of the gene-off experiment, the majority of the neurons displayed nuclear staining. If all of these neurons were to die, a further decrease in striatal area would result during the gene-off period. Instead, this does not occur, and if anything, a mild recovery takes place. Consistent with these findings are the other mouse models that display nuclear staining and aggregates, but no cell death (Davies et al., 1999; Usdin et al., 1999; Wheeler et al., 1999). Whether or not the polyQ-htt is toxic in an aggregated or soluble state, we demonstrate that its continuous expression is required for the progression of the pathology.

The decrease in reactive astrogliosis indicates that there is a decrease of the toxic insult exerted by the

expression of the polyQ-htt. When expression is discontinued, this toxic insult also ceases. It is at this point that the brain can recover some of its functions, which is attested to by the amelioration of the clasping phenotype. Preliminary studies on the rotarod further support recovery. Although 18-week-old gene-on HD94 mice were significantly impaired at this task and failed to maintain their balance, gene-off HD94 mice regained their ability to maintain their balance on the rotarod and were indistinguishable from control. We are currently investigating potential physiologic changes that may underlie recovery by using cellular recording and more global measurements, such as 2-deoxyglucose and PET scans, before and after turning gene expression off.

One of the most puzzling characteristics of HD is the striatal-specific atrophy despite widespread expression of htt. Two of the most common explanations are that either the striatum itself is particularly vulnerable to the expression of polyQ-htt, or the cortex is vulnerable to the polyQ-htt and then exerts a detrimental effect on the striatum. We have shown here that expression of polyQ-htt in the forebrain is sufficient to recapitulate key neuropathological features of HD. To further explore the spatial relationship between expression of polyQ-htt and HD, we will take advantage of the binary nature of the system we have employed to create the HD94 mice. We are currently expressing polyQ-htt in either the striatum or cortex to address this issue.

In summary, we have shown that turning off the expression of polyQ-htt at an age when HD94 mice are displaying neuropathological and behavioral phenotypes results in either a halt or a reversion of the different phenotypes. This implies that irreversible changes that commit the cell to neuronal dysfunction or death have not necessarily taken place. Our results therefore suggest that therapeutic approaches aimed either to destroy or inactivate the mutant huntingtin protein might be effective. Furthermore, the motor recovery indicates that plastic changes can occur when the toxic insult ceases. The elucidation of the mechanisms responsible may provide new targets for therapeutic interventions for patients suffering from HD and other neurodegenerative disorders.

Experimental Procedures

Generation of Injection Fragment

The microinjection construct was a 1.1 kb NarI-XhoI fragment that consists of ~100 bp UTR sequences, a chimeric mouse/human exon 1 with a polyQ expansion of 94 repeats, with an A to G mutation in the 42nd CAG triplet, and ~600 bp of intronic sequence. The chimeric mouse/human exon 1 fragment was created by replacing a XmnI-KpnI fragment from a 1.6 kb PstI mouse genomic fragment with an XmnI-KpnI PCR generated human fragment (received from Drs. S. Zeitlin and A. Efstratiadis) (Levine et al., 1999). The NarI-XhoI fragment was reoriented by subcloning into ClaI-XhoI of pBSII KS(+) (Stratagene). Next, a 1 kb PstI-XhoI was cloned into PstI-Sall of a plasmid containing a bidirectional tetO sequence flanked by cytomegalovirus (CMV) minimal promoters with lacZ reporter sequences (pBI-3) (Baron et al., 1995). Lastly, a 7.7 kb *Asel* fragment was microinjected into single cell CBAx57BL/6 embryos. The founder male was crossed with wild-type (wt) CBAx57BL/6 females. Southern analysis of the founder and F1 progeny revealed integration of one copy of the injection fragment (data not shown).

Animals

All experiments were performed on CBAx57BL/6 inbred mice. BiTetO, CamKII α -tTA, HD94, and wt mice were bred at the New York State Psychiatric Institute animal facility. Mice were housed five per cage with food and water available ad libitum. They were maintained in a temperature-controlled environment on a 12 hr light/dark cycle with light onset at 6:00 a.m. Mice on doxycycline treatment were housed up to five mice per cage, but were given 2 mg/ml dox in a 5% sucrose solution instead of the normal drinking water. Dox solution was maintained in dark bottles and changed once a week. Mice were derived from heterozygote crosses to ensure obtaining all genotypes in each litter.

Tail Suspension Test

Mice from ages 3 to 40 weeks were suspended by their tails for 15 s and videotaped. Animals were assessed for clasping score and clasping duration. For clasping score, the video was played back in half-time. The test period was divided into 2 s slots. An animal would receive a score of 1 point if they displayed any abnormal movement during the given time slot, therefore allowing for a maximum score of 7 points. An abnormal movement was defined as any dystonic movement of the hindlimbs, or a combination of hind- and forelimbs and trunk, during which the limbs were pulled into the body in a manner not observed in wt mice.

Tissue Preparation and Histology

Fresh frozen coronal sections were obtained from brains isolated from P1 and adult mice. 20 to 30 μ m sections were cut onto slides on a cryostat. Perfused-fixed coronal sections were generated by transcardial perfusion with 4% paraformaldehyde in PB. Brains were removed, post-fixed for 1 hr at 4°C, and cryoprotected overnight in a 30% sucrose solution in 0.1 M PB. 20 μ m sections were cut on a cryostat and thaw-mounted onto slides or generated for free-floating. LacZ staining was performed as follows: Fresh frozen sections were postfixed for 10 min in 4% paraformaldehyde in Soren's buffer. Slides were then incubated for 1 hr at 30°C in LacZ staining solution (1 mg/ml X-gal [4-chloro-5-bromo-3-indolyl- β -galactosidase, Boehringer Mannheim], 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM MgCl₂ in PBS). After staining, sections were counterstained with fast red (SIGMA), rinsed, and dry-mounted. Immunohistochemistry was performed on both fresh frozen and fixed sections. A minimum of three mice per age per group were used for each experiment. 20 μ m fresh frozen sections were postfixed for 1 hr. Sections obtained from perfused brain tissue were either thaw-mounted or free-floating, and were processed as previously described (Lucas et al., 1998). Antibodies were used at the following concentrations: glial fibrillary acid protein (GFAP 1:1000, Incstar), Ubiquitin (1:500, Dako), and HD1 (1:2000) (Davies et al., 1997). HD1 stained sections were counterstained with cresyl violet (Nissl staining).

Radioligand Binding

[³H]-SCH 23969 binding: 20 μ M fresh frozen sections were preincubated in Tris buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, and 1 mM MgCl₂ [pH 7.4]) at room temperature (rt). Sections were then incubated in buffer containing 2 nM [³H]-SCH 23969 (Schering Plough) for 90 min at rt. Incubation buffer also contained 30 nM spiperone HCl dissolved in ethanol to mask 5-HT₂ and D₂ binding sites. Nonspecific binding was assessed using 10 μ M flupentixol in the incubation buffer. Sections were exposed to [³H]-sensitive film (Hyperfilm, Amersham International) for two weeks. Analysis: Autoradiographic film images were digitized and analyzed with NIH Image 160/ppc. For each measurement of binding, background measurements were taken from corresponding nonspecific binding sections, and subtracted from each respective reading before analysis. Four matched sections per brain were analyzed by repeated-measures ANOVA, followed by Fisher post-hoc tests.

Brain Measurements

A series of measurements (anterior to posterior) were taken on matched sections per brain. Three matched sections were used for striatal area measurements. Measurements were taken in units of

square micrometers by Stereoimager. The measure was then converted to percent control. GFAP and nuclear counts were determined across four matched striatal sections from three brains per group. Successive measures were then analyzed by repeated-measures ANOVA followed by Fisher post-hoc tests.

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